

# Wnt/ $\beta$ -Catenin Signaling Regulates Proliferation of Human Cornea Epithelial Stem/Progenitor Cells

Martin N. Nakatsu,<sup>1,2</sup> Zhenhua Ding,<sup>1,2</sup> Madelena Y. Ng,<sup>1</sup> Thuy T. Truong,<sup>1</sup> Fei Yu,<sup>3</sup> and Sophie X. Deng<sup>1</sup>

**PURPOSE.** To investigate the expression and role of the Wnt signaling pathway in human limbal stem cells (LSCs).

**METHODS.** Total RNA was isolated from the human limbus and central cornea. Limbal or cornea-specific transcripts were identified through quantitative real-time PCR. Protein expression of Wnt molecules was confirmed by immunohistochemistry on human ocular tissue. Activation of Wnt signaling using lithium chloride was achieved in vitro and its effects on LSC differentiation and proliferation were evaluated.

**RESULTS.** Expression of Wnt2, Wnt6, Wnt11, Wnt16b, and four Wnt inhibitors were specific to the limbal region, whereas Wnt3, Wnt7a, Wnt7b, and Wnt10a were upregulated in the central cornea. Nuclear localization of  $\beta$ -catenin was observed in a very small subset of basal epithelial cells only at the limbus. Activation of Wnt/ $\beta$ -catenin signaling increased the proliferation and colony-forming efficiency of primary human LSCs. The stem cell phenotype was maintained, as shown by higher expression levels of putative corneal epithelial stem cell markers, ATP-binding cassette family G2 and  $\Delta$ Np63 $\alpha$ , and low expression levels of mature cornea epithelial cell marker, cytokeratin 12.

**CONCLUSIONS.** These findings demonstrate for the first time that Wnt signaling is present in the ocular surface epithelium and plays an important role in the regulation of LSC proliferation. Modulation of Wnt signaling could be of clinical application to increase the efficiency of ex vivo expansion of corneal epithelial stem/progenitor cells for transplantation. (*Invest Ophthalmol Vis Sci.* 2011;52:4734–4741) DOI:10.1167/iops.10-6486

The corneal epithelium is constantly renewed and maintained by corneal epithelial stem cells, or limbal stem cells (LSCs), that are presumed to reside at the limbus, the junction between the cornea and conjunctiva.<sup>1,2</sup> LSCs undergo infrequent division and give rise to transient amplifying cells (TACs) that continue to proliferate and migrate centripetally and apically to maintain the normal homeostasis of the corneal epithelium.<sup>3,4</sup> These stem cells are characterized by a high capacity of self-renewal and slow cycling in normal physiological

conditions, but they exhibit high proliferative potential during wound healing and in tissue culture.<sup>5–7</sup>

Despite the success of corneal surface reconstruction by transplanting ex vivo expanded LSCs in humans,<sup>8–11</sup> the external and intrinsic signaling pathways that govern the self-renewal and differentiation of LSCs remain largely unknown. Several studies suggest that the extracellular microenvironment/niche of LSCs appears to control their plasticity as in other stem cells.<sup>12–14</sup> For example, differentiated corneal epithelium becomes dedifferentiated when cultured on limbal stroma in vitro, whereas less differentiated limbal epithelial cells become differentiated on corneal stroma.<sup>15</sup> In addition, the outgrowths from limbal explants lose stem cell properties when they migrate further away from explant tissues that hold the LSC niche.<sup>16</sup> Successful transdifferentiation of hair follicle stem cells into cornea-like epithelial cells under a corneal limbal microenvironment further underscores the importance of niche factors in stem cell differentiation.<sup>17</sup>

Wnt signaling is very complex and there are 19 Wnt proteins, 10 Frizzled (Fzd) receptors, 4 Dickkopf (Dkk) inhibitors, and several other Wnt inhibitory proteins that are currently known to modulate the pathway. The Wnt pathway has been implicated in the regulation of self-renewal and cell fate determination in embryogenesis and stem cells from a variety of tissues.<sup>18–20</sup> Wnt signaling is important in the development of ocular tissues as well. Activation of canonical Wnt signaling promotes the formation of retina in mice, whereas expression of specific Wnt proteins and Fzd receptors in lens during embryonic development indicates their function in lens epithelium and lens fiber differentiation.<sup>21–25</sup> Wnt4 expression was detected in human fetal limbal epithelial cells and in the adult limbal region<sup>26</sup>; lymphoid enhancer-binding factor 1 and frizzled-related protein (FRZB) were upregulated in limbal epithelial cells within the limbal epithelial crypt structure.<sup>27</sup> Interestingly, Dkk2 regulates cell fate determination and Wnt/ $\beta$ -catenin activity is required for proper development of the ocular surface epithelium in mice.<sup>28</sup> Nuclear localization of  $\beta$ -catenin is also observed in actively dividing limbal basal epithelial cells that invade the limbal stroma in explant cultures.<sup>29</sup> Our previous results showed that expression of Wnt inhibitory factor 1 (WIF1) is greater in the limbus than that in the conjunctiva and cornea in vervet monkeys.<sup>30</sup> These observations suggest a possible role for Wnt signaling in LSC regulation.

In the study described here, we further investigated the Wnt signaling pathway in human LSCs. Quantitative real-time polymerase chain reaction (qRT-PCR) arrays revealed that different Wnt genes were preferentially upregulated in the limbal region compared with expression in the central cornea. Activation of Wnt/ $\beta$ -catenin signaling promoted LSC proliferation and increased colony-forming efficiency (CFE), and the progenitor/stem cell state of the treated LSCs was maintained. Taken together, these findings indicate an important role for Wnt signaling in the regulation of human LSC proliferation and, possibly, differentiation.

From the <sup>1</sup>Cornea and Uveitis Division, Jules Stein Eye Institute, and the <sup>3</sup>Department of Biostatistics, University of California Los Angeles, Los Angeles, California.

<sup>2</sup>These authors contributed equally to the work presented here and should therefore be regarded as equivalent authors.

Supported by the Emily G. Plumb Estate and Trust and National Eye Institute Grant 5T32EY007026-35 (MNN).

Submitted for publication August 28, 2010; revised November 2, 2010; accepted January 10, 2011.

Disclosure: M.N. Nakatsu, None; Z. Ding, None; M.Y. Ng, None; T.T. Truong, None; F. Yu, None; S.X. Deng, None

Corresponding author: Sophie X. Deng, Cornea and Uveitis Division, Jules Stein Eye Institute, UCLA, 100 Stein Plaza, Los Angeles, CA 90095; deng@jsei.ucla.edu.

## METHODS

### Human Sclerocorneal Tissue

Human sclerocorneal tissues from healthy donors were obtained from the Lions Eye Institute for Transplant and Research (Tampa, FL), the Tissue Bank International (Baltimore, MD), or the San Diego Eye Bank (San Diego, CA). Experimentation on human tissue adhered to the tenets of the Declaration of Helsinki. The experimental protocol was evaluated and approved by the UCLA Institutional Review Boards. Only those tissues that had intact limbal and corneal epithelium were selected for this study. The ages of the donors ranged from 20 to 65 years. For the purpose of RNA isolation, the death to preservation time was <7 hours. The tissues were either snap frozen on dry ice on procurement or preserved in solution (Optisol; Chiron Ophthalmics, Inc., Irvine, CA) for <72 hours at 4°C. For immunohistochemistry, death to preservation time was <10 hours and time to tissue processing was <6 days. For the purpose of cell culture, the limbal rim was collected from the unused portion of healthy donor tissues immediately after cornea transplantation.

### Primary Limbal Epithelial Cell Culture

Human limbal epithelial cells were isolated from the limbus of eye bank corneas after the central cornea button was used for transplantation. The residue iris tissue, endothelium, and conjunctiva were surgically removed. The tissues were treated with 2.4 U/mL of a grade II neutral protease (Dispase II; Roche, Indianapolis, IN) in keratinocyte serum-free medium (KSFM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS) (Invitrogen) at 37°C for 2 hours. The limbal epithelium layer was isolated under a dissecting microscope. Single-cell suspensions were collected after treatment with 0.25% trypsin (Invitrogen) for 30 minutes. The limbal epithelial cells were seeded on growth-arrested NIH 3T3 feeder layers obtained from the American Type Culture Collection (ATCC, Manassas, VA) at a density of  $1 \times 10^4$  cells/cm<sup>2</sup> in KSFM supplemented with 10% FBS, bovine pituitary extract, and 10 ng/mL of epidermal growth factor.

To prepare growth-arrested feeder layers, subconfluent 3T3 cells were incubated with 16 µg/mL mitomycin C (Sigma-Aldrich, St. Louis, MO) for 2 hours at 37°C and then trypsinized. The mitomycin C-treated cells were subcultured in Dulbecco's modified Eagle's medium (DMEM; ATCC), supplemented with 10% FBS at a density of  $2 \times 10^4$  cells/cm<sup>2</sup>. Lithium chloride (LiCl; Sigma-Aldrich) at a final concentration of 500 µM was added to the culture medium of the treatment group. The media was changed every 2 to 3 days and the length of time for cell culture was between 14 and 21 days.

To assess the CFE of limbal epithelial cells, the culture dishes were fixed and stained with 0.5% rhodamine B (Sigma-Aldrich) for 10 minutes. Colonies were counted under a dissecting microscope. CFE was calculated by dividing the number of colonies by the number of limbal epithelial cells seeded. The average from five independent experiments was obtained and the *P* value was calculated by using the Student's *t*-test.

### RNA Extraction and qRT-PCR

The corneal and limbal epithelia along with their immediate adjacent stroma were dissected from human sclerocorneal tissues as described previously.<sup>30</sup> All tissue and cell culture samples were stored at -80°C until RNA extraction was performed. To ensure complete homogenization of tissue samples, a serrated homogenizer (Omni International, Marietta, GA) was used. Between periods of homogenization, the probe was rinsed twice with filtered distilled deionized (ddI) water (Millipore, Billerica, MA), once with 70% ethanol and twice again in fresh ddI water. When RNA was isolated from cell culture samples, the cultured cell layer was treated with 1 mg/mL of collagenase I (Sigma-Aldrich) for 30 minutes at 37°C. The colonies were detached from the 3T3 feeder layer and collected with forceps under a dissecting microscope. The isolated colonies were lysed and homogenized using a biopolymer shredding system (QIAshredder; Qiagen, Valencia, CA). Total RNA from

tissues and cell culture samples was then extracted (Qiagen RNeasy Mini Kit; Qiagen). The quantity and quality of total RNA were assessed by spectrophotometer (NanoDrop 1000; NanoDrop, Wilmington, DE) and bioanalyzer (2100 Bioanalyzer; Agilent Technologies, Santa Clara, CA). Only those samples that had an RNA integrity number > 9 and exhibited minimal RNA degradation were used for subsequent experiments.

Total RNA was reverse-transcribed using RNase H2 reverse transcriptase (RT; SuperScript II, Invitrogen) in triplicate, according to the manufacturer's recommendations. The relative abundance of transcripts was detected through qRT-PCR using a PCR program mix (Brilliant SYBR Green QPCR Master Mix; Stratagene, La Jolla, CA). The protocol used a real-time PCR system (Mx3000p; Stratagene). Cycling conditions were as follows: an initial denaturing step of 5 minutes at 94°C and subsequent 40 cycles of amplification in which each cycle consisted of 15 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 72°C. To generate a dissociation curve after the amplification cycles, each sample was incubated at 95°C for 1 minute followed by a melting curve program (55–99°C, with a 5-second hold at each temperature). The fluorescence intensity of each sample was acquired during the execution of the melting curve program and normalized in relation to that of the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase. The average value of the triplicates from each transcript was used for comparison. At least three independent experiments were performed on each donor and a total of three donors were used for the qRT-PCR. To allow for direct comparison among independent experiments, the ratio of the absolute expression values at the limbus to that of the cornea was used. Any transcript with an expression ratio  $\geq 2$  was considered specific to the limbal region, whereas a ratio  $\leq 0.5$  was considered specific to the cornea. The primers used for qRT-PCR are listed in Table 1.

TABLE 1. qRT-PCR Primers

Gene	Direction	Primer Sequence
<i>Wnt2</i>	Forward	CCCCCTCTCGGTGGAATCTGGCTC
	Reverse	GCTGTGATCCCTGTCCAGGTGTT
<i>Wnt3</i>	Forward	CGAGTCGGCCTTCGTTTAC
	Reverse	CGAGTCACAGCCGCAATG
<i>Wnt6</i>	Forward	GGCTGTGGGCGCCCTTGTTAT
	Reverse	CGAGGTAGCTTGCACCACT
<i>Wnt7a</i>	Forward	CATAGGAGAAGGCTCACAATGG
	Reverse	CGGCAATGATGGCGTAGGT
<i>Wnt7b</i>	Forward	CACAGAACTTTCCGAAGTGG
	Reverse	GGCTAGGCCAGGAATCTTGT
<i>Wnt10a</i>	Forward	ACACAGTGTGCTTACATTGC
	Reverse	TGGATGGCGATCTGGATGC
<i>Wnt11</i>	Forward	CTGACCTCAAGACCCGATACC
	Reverse	CGAGTTCGAGTCCCTTCACAG
<i>Wnt16b</i>	Forward	GCCCAAGGAACTGGATGTG
	Reverse	GCTCCCGCACTCCTGAATG
<i>Dkk-1</i>	Forward	CATCAGACTGTGCCTCAGGA
	Reverse	CCACAGTAACAACGCTGGAA
<i>WIF1</i>	Forward	AGGAGAGCCTGTACCTATGGA
	Reverse	TTCTCTGTTGCGCTTTTCTGA
<i>FRZB</i>	Forward	TCCCGAGGCCATCGTTACT
	Reverse	AGGCTTACATTTACAGCGTTTAC
<i>SFRP-5</i>	Forward	TGCTGCACTGCCACAAGTT
	Reverse	GTGCTCCATCTCACACTGGG
<i>Ki67</i>	Forward	CTTTGGGTGGCACTTGACG
	Reverse	GTGACCCCGCTCCTTTT
<i>ABCG2</i>	Forward	CCGCGACAGCTTCCAAATGACCT
	Reverse	GCCGAAGAGCTGCTGAGAACTGTA
<i>ΔNp63a</i>	Forward	TCCATGGATGATCTGGCAAGT
	Reverse	GCCCTTCCAGATCGCATGT
<i>K12</i>	Forward	CCAGGTGAGTCCAGGTAGAA
	Reverse	CCTCCAGGTTGCTGATGAGC

Dkk-1, Dickkopf 1; WIF1, Wnt inhibitory factor 1; SFRP-5, secreted frizzled-related protein 5; ABCG2, ATP-binding cassette subfamily G member 2; K, cytokeratin.

## Immunohistochemistry

The human sclerocorneal tissues obtained from eye banks were cut into four quadrants and embedded in optimal cutting temperature compound (Sakura Finetek, Torrance, CA) on dry ice. Tissues were cut into 8- $\mu$ m sections by using a cryostat (Leica CM3050S; Leica Microsystems, Wetzlar, Germany) and stored at  $-80^{\circ}\text{C}$  until they were used. Limbal epithelial cells cultured on mouse 3T3 feeder layers on chamber slides (Laboratory-Tek; Nunc, Rochester, NY) for 20 days were used for immunostaining. The cell culture slides or tissue cryosections were fixed in 4% formaldehyde for 20 minutes and permeabilized by washing three times for 5 minutes with 0.3% Triton X-100 (Sigma-Aldrich) in PBS (Invitrogen). Fixed slides were incubated with 5% blocking serum (Jackson ImmunoResearch Laboratories, West Grove, PA) in 1% BSA in PBS (Invitrogen) for 30 minutes, washed three times with 1% BSA/PBS, and incubated with primary antibodies for 1 hour at room temperature or at  $4^{\circ}\text{C}$  overnight. The slides were then washed three times with 1% BSA/PBS and labeled with the appropriate secondary antibody for 1 hour at room temperature. Primary and secondary antibodies used are summarized in Table 2. The nuclei were labeled with Hoechst 33342 nucleic acid stain (Invitrogen) at 0.5  $\mu\text{g}/\text{mL}$  for 10 minutes. The slides were then washed with PBS three times and mounted in gel mount medium (Sigma-Aldrich). Pictures were taken under a  $25\times$  objective using a fluorescent light microscope (Carl Zeiss Inc., Oberkochen, Germany) or confocal microscope (Leica TCS-SP; Leica Microsystems).

## Statistical Analysis

To eliminate the variation between experiments in the qRT-PCR, treatment-to-control ratio values were obtained from each of the three independent experiments and averaged. A Student's *t*-test was performed on the ratio values. Bar graphs represent means  $\pm$  SEM from three separate experiments. A value of  $P < 0.05$  was considered statistically significant.

## RESULTS

### Expression of Wnt Signaling in the Human Limbus

Total RNA was extracted from the human limbus and central cornea, respectively. As an additional quality control measure for our RNA samples, we analyzed the expression patterns of known markers in the cornea and limbus in each of the RNA samples isolated from three different donors. Cytokeratin (K) 12 is a specific marker of mature cornea epithelial cells, whereas K15 is predominantly expressed in the limbal and conjunctival epithelium.<sup>2,31</sup> Expression levels of these two markers were quantified by qRT-PCR. K12 transcripts were predominantly expressed in the cornea but minimally in the limbus, whereas K15 expression was restricted to the limbus

(data not shown). The expression levels of these two signature genes correlate well with the expected expression pattern and further validate the quality and the anatomic source of the RNA.

To detect whether the Wnt signaling pathway was present on the ocular surface, expression of Wnt signaling-related genes in the human limbus and cornea was first screened by using a PCR array of the Wnt signaling pathway. Several Wnt activators and inhibitors were specific to either the limbus or the cornea (data not shown). qRT-PCR was performed to further confirm the above-mentioned preferential expression profile (Fig. 1A). Wnt2, Wnt6, Wnt11, Wnt16b, Dickkopf 1 (Dkk-1), FRZB, secreted frizzled-related protein 5 (SFRP-5), and WIF1 were all preferentially expressed in the limbus, whereas Wnt3, Wnt7a, Wnt7b, and Wnt10a expression were upregulated in the cornea. There were significant differences among the expression levels of the above-cited transcripts in the limbus and cornea, respectively ( $P < 0.05$ ). In addition, RT-PCR confirmed the specificity of the primers for the preferential Wnt transcripts by showing a single band of the predicted size (Fig. 1B).

Expressions of  $\beta$ -catenin and two specific Wnt transcripts in the limbal region were confirmed at the protein level by immunohistochemistry on human sclerocorneal tissues. Confocal microscopy showed that  $\beta$ -catenin was primarily located at the cell membrane in all epithelial cells, but was also present in the cytoplasm and nucleus in a small group of limbal epithelial cells in the basal and suprabasal layers (Figs. 1C, 1D). White arrows highlight nuclear localization of  $\beta$ -catenin (Fig. 1D, inset). Membrane-bound  $\beta$ -catenin was evenly expressed in all layers of the corneal epithelium (Fig. 1C). No nuclear localization of  $\beta$ -catenin was observed in the corneal epithelium.

Wnt2 was expressed mainly at the superficial and suprabasal layers and, to a lesser extent, in the basal limbal layer of the epithelium, but was barely detected in the corneal epithelium (Figs. 1E, 1F). Wnt16 was predominantly localized to the suprabasal and basal layers of the limbal epithelium. In contrast, expression in the central cornea was limited to the superficial epithelial layer (Figs. 1G, 1H).

Overall, we demonstrate that different Wnt signaling-related genes were preferentially expressed in the human limbus or cornea. This finding suggests a role for Wnt signaling in the regulation of corneal epithelial cell proliferation and differentiation.

### Activation of Wnt Signaling Promotes Proliferation of Corneal Epithelial Stem/Progenitor Cells

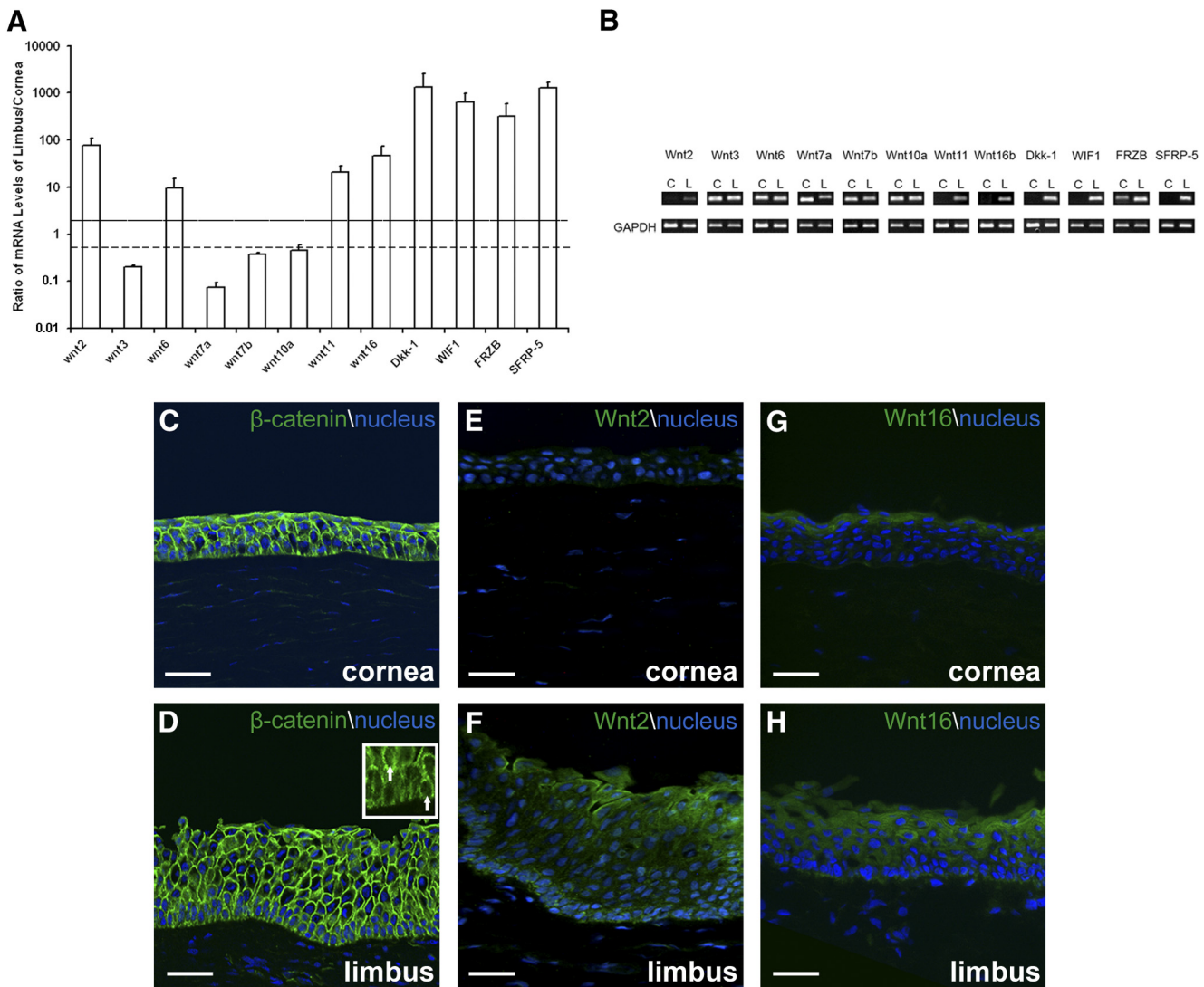
To investigate the function of the Wnt signaling pathway in LSCs, we used LiCl, a well-established activator of Wnt/ $\beta$ -

TABLE 2. Primary and Secondary Antibodies

Protein	Company	Host	Species Reactivity
Ki67	Dako, Carpinteria, CA	Mouse	Human
$\beta$ -catenin	BD Biosciences, San Jose, CA	Mouse	Human
Cytokeratin 12	Santa Cruz Biotechnology, Santa Cruz, CA	Goat	Human
BCRP/ABCG2	Abcam, Cambridge, MA	Rat	Human/mouse
p63 $\alpha$	Cell Signaling Technology, Danvers, MA	Rabbit	Human
Wnt2	Santa Cruz Biotechnology, Santa Cruz, CA	Goat	Human
Wnt16	Santa Cruz Biotechnology, Santa Cruz, CA	Rabbit	Human
Alexa Fluor 488 IgG	Invitrogen, Carlsbad, CA	Goat	Rabbit
Alexa Fluor 488 IgG	Invitrogen, Carlsbad, CA	Goat	Mouse
Alexa Fluor 488 IgG	Invitrogen, Carlsbad, CA	Goat	Rat
Alexa Fluor 488 IgG	Invitrogen, Carlsbad, CA	Donkey	Goat

BCRP/ABCG2, breast cancer resistance protein/ATP-binding cassette sub-family G member 2; IgG, immunoglobulin G.





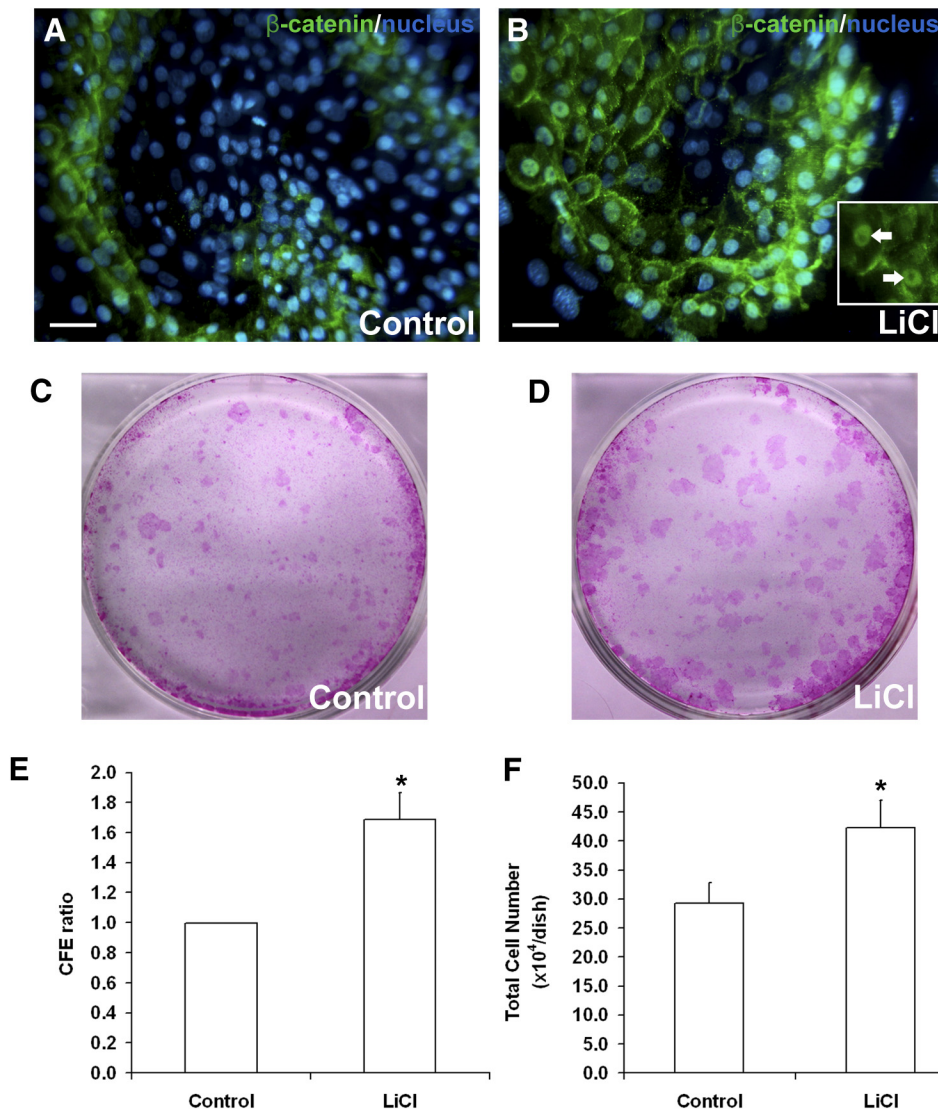
**FIGURE 1.** Endogenous Wnt expression in the human cornea and limbal epithelium. **(A)** Expression level of several Wnt and Wnt-related transcripts quantified by qRT-PCR. The ratio of the expression level in the limbus versus cornea was used. A ratio  $\geq 2$  was considered limbal-specific and  $\leq 0.5$  was corneal-specific. Wnt3, Wnt7a, Wnt7b, and Wnt10a were upregulated in the cornea, whereas Wnt2, Wnt6, Wnt11, Wnt16, Dickkopf 1 (Dkk-1), Wnt inhibitory factor 1 (WIF1), FRZB, and secreted frizzled-related protein 5 (SFRP-5) were overexpressed in the limbus. **(B)** RT-PCR confirmed the predicted size for these Wnt transcripts. **(C, D)** Confocal microscopy confirmed  $\beta$ -catenin localization at the cell membrane in both the limbal and corneal epithelium.  $\beta$ -Catenin was also observed in the cytoplasm and nucleus of select number of cells in the basal and suprabasal layers of the limbus. *White arrows (inset)* highlight  $\beta$ -catenin localization in the nucleus of basal layer epithelial cells. **(E, F)** Wnt2 was highly expressed at the limbal superficial and suprabasal layers and, to a lesser extent, at the basal epithelium. Expression was absent in the cornea epithelium. **(G, H)** Wnt 16 was expressed at the limbal suprabasal and basal epithelium layers. Expression in the central cornea was limited to the superficial epithelium. Scale bar, 50  $\mu$ m.

catenin signaling,<sup>32,33</sup> by inhibiting glycogen synthase kinase 3- $\beta$ , to activate Wnt/ $\beta$ -catenin signaling in vitro. Freshly isolated human limbal epithelial cells in single-cell suspension were cultured for 20 days in the presence or the absence of LiCl. No increase in cell death was observed in the LiCl-treated cell cultures by gross examination.

In the absence of LiCl,  $\beta$ -catenin was mainly membrane bound or in the cytoplasm of a very small group of cells (Fig. 2A). In the presence of LiCl,  $\beta$ -catenin was detected in the nucleus, demonstrating nuclear translocation and indicative of Wnt/ $\beta$ -catenin signaling activation (Fig. 2B, inset).

When Wnt signaling was activated with LiCl, the number and size of the colonies were significantly greater (Figs. 2C, 2D). The CFE ratio in the LiCl-treated groups was 1.7-fold higher than that in the control groups based on five independent experiments (Fig. 2E) ( $P < 0.05$ ). The CFE ratio was obtained by dividing the

CFE of the LiCl-treated cultures by that from the control, which was then set to 1. In two representative experiments, colonies were also isolated from the feeder layer and the total number of expanded epithelial cells was counted. LiCl treatment yielded an average of  $4.2 \pm 0.5 \times 10^5$  total limbal epithelial cells on each 30-mm culture dish and the number of epithelial cells in the control was only  $2.9 \pm 0.3 \times 10^5$ . This result indicated a 1.4-fold increase in the total cell number with LiCl treatment (Fig. 2F) ( $P < 0.05$ ). Furthermore, to confirm that LiCl increased the proliferation of LSCs, Ki67, a proliferation marker, was used to detect actively proliferating cells. Immunohistochemistry showed that there were more Ki67<sup>+</sup> cells in LiCl-treated cultures (Fig. 3B) than those in the control (Fig. 3A). The level of Ki67 expression was further quantified by qRT-PCR (Fig. 3C). We detected a 2.9-fold increase in the expression of Ki67 in the LiCl-treated LSCs compared with that in the control ( $P < 0.05$ ). These findings indicate



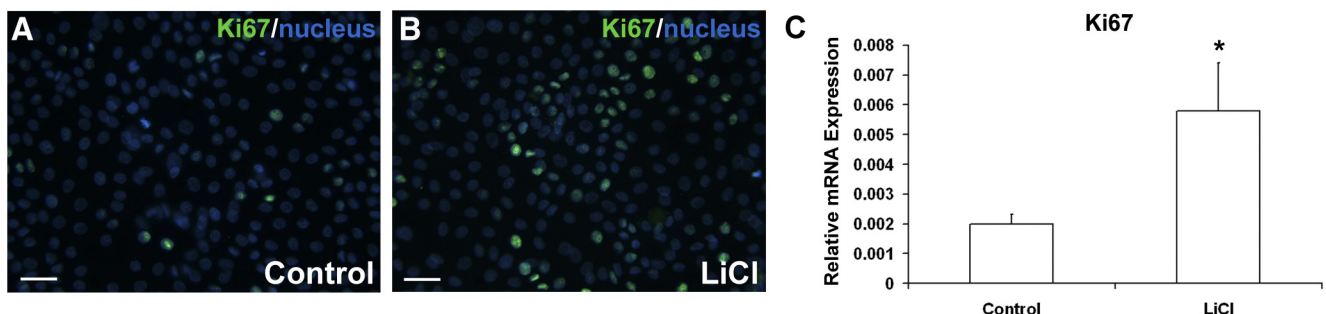
**FIGURE 2.** Activation of Wnt/ $\beta$ -catenin signaling using LiCl in primary limbal epithelial stem/progenitor cells cultured on the 3T3 feeder layer. (A, B) Localization of  $\beta$ -catenin in the control and LiCl-treated LSCs. In the absence of LiCl,  $\beta$ -catenin was expressed predominantly on the cell membrane (A). In the presence of LiCl, nuclear localization of  $\beta$ -catenin was observed (B). White arrows (inset) highlight  $\beta$ -catenin nuclear localization in cultured LSCs. (C–F) Effect of LiCl on CFE and cell proliferation. In the LiCl-treated dish (D), colonies were larger and greater in number compared with the control (C). The CFE ratio was 1.7-fold higher in the LiCl-treated cultures compared with control (E). The total number of limbal epithelial cells showed a 1.4-fold increase in the presence of LiCl (F). \* $P < 0.05$ . Scale bar, 20  $\mu$ m.

that activation of the Wnt/ $\beta$ -catenin signaling pathway promotes the proliferation of limbal epithelial stem/progenitor cells in vitro.

### Corneal Epithelial Stem/Progenitor Cells Remain Undifferentiated When Wnt/ $\beta$ -Catenin Signaling Is Activated

To examine whether activation of Wnt/ $\beta$ -catenin signaling alters the differentiation status of cornea stem/progenitor cells

in single-cell suspension cultures, expression of two putative LSC markers, ATP-binding cassette subfamily G member 2 (ABCG2)<sup>34,35</sup> and  $\Delta$ Np63 $\alpha$ , were evaluated. Previously, Pellegrini et al.<sup>36</sup> showed that p63 was highly expressed in the basal epithelium of the human limbus, but was not detected in the central cornea. This marker was also able to differentiate human keratinocyte stem cells from transamplifying keratinocytes. Furthermore, the alpha isoform, p63 $\alpha$ , was proposed to



**FIGURE 3.** Activation of Wnt/ $\beta$ -catenin signaling increased the proliferation of limbal epithelial stem/progenitor cells. (A, B) An increase in Ki67<sup>+</sup> cells was observed in the LiCl-treated cultures (B) compared with control (A). (C) Ki67 expression level was quantified by qRT-PCR. \* $P < 0.05$ . Scale bar, 20  $\mu$ m.



be a more specific limbal epithelial stem cell marker than all other isoforms of p63, and  $\Delta Np63\alpha$  has been shown to be expressed only in the basal to intermediate layers of the limbal epithelium.<sup>37–39</sup> Expression of K12 was used to detect fully differentiated corneal epithelial cells. Immunostaining showed that high levels of both ABCG2 and  $\Delta Np63\alpha$  were expressed in cultured limbal epithelial cells in the control and in the presence of LiCl (Figs. 4A–D), whereas K12 expression was detected in a smaller population of cells in the LiCl-treated group compared with the control (Figs. 4E, 4F). A primer that specifically amplifies  $\Delta Np63\alpha$  was used to detect this marker's mRNA expression level. Consistent with the observation from the immunostaining experiment, ABCG2 expression was maintained,  $\Delta Np63\alpha$  expression was significantly higher, and K12 expression was significantly lower when Wnt/ $\beta$ -catenin signaling was activated (Fig. 4G). These results demonstrate that activation of Wnt/ $\beta$ -catenin signaling maintains the undifferentiated phenotype of cultured LSCs.

## DISCUSSION

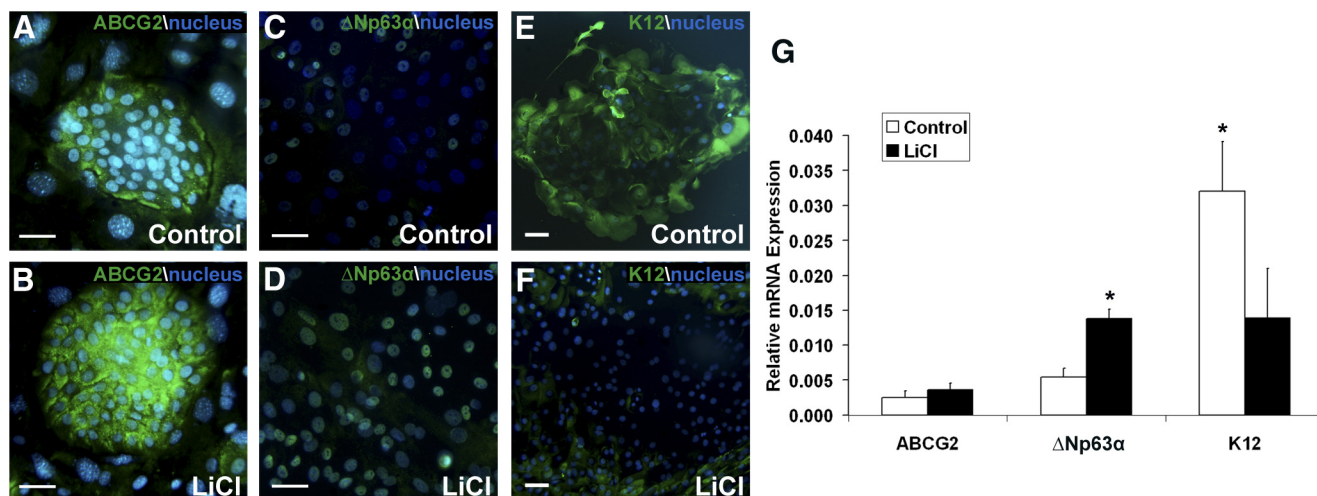
This is the first comprehensive study to investigate the Wnt signaling pathway on the human ocular surface epithelium. We first showed that  $\beta$ -catenin is expressed in normal human limbal and corneal epithelial cells. This observation was consistent with previous findings.<sup>29,40</sup> Of the Wnt molecules that we profiled, Wnt2, Wnt6, Wnt11, and Wnt16b were overexpressed in the human limbus where corneal epithelial stem cells are located, whereas Wnt3, Wnt7a, Wnt7b, and Wnt10a were specific to the mature corneal epithelium. Interestingly, Wnt7a expression rapidly increased in the central and peripheral regions of the human corneal epithelium during wounding, where TACs and terminally differentiated cells reside, whereas  $\beta$ -catenin nuclear localization was observed in corneal epithelial cells at the leading edge of the wound.<sup>40,41</sup> These data suggest that during normal homeostasis of the corneal epithelium, Wnt/ $\beta$ -catenin signaling may be relatively inactive and can be stimulated during wound healing. This is consistent with our finding that  $\beta$ -catenin is mainly membrane bound in the normal intact corneal epithelium (Fig. 1C). FRZB was shown to be upregulated in limbal epithelial cells within the

limbal epithelial crypt structure compared with the cornea, which is consistent with our findings.<sup>27</sup>

Wnt signaling has diverse effects on various stem cells. In mouse and human embryonic stem cells, activation of the Wnt pathway sustains self-renewal of human embryonic stem cells.<sup>42–45</sup> In adult tissues, specific Wnt proteins regulate self-renewal and differentiation of neural stem cells,<sup>46,47</sup> skin stem cells,<sup>48–50</sup> and intestinal stem cells.<sup>51,52</sup> The observation that some Wnt proteins regulate self-renewal and others regulate differentiation suggests that the effects of Wnt molecules likely depend on the intrinsic responsiveness of the target cells and the niche factors under physiological conditions.<sup>42,53–56</sup> Our previous finding showing that the limbal region has a different transcriptional profile compared with that of the cornea supports this notion.<sup>30</sup> The exact role of each of these limbal-specific Wnt molecules in the regulation of self-renewal or differentiation of corneal epithelial progenitor cells will need further investigation.

The expression of  $\beta$ -catenin, which is critical in cell adhesion junctions<sup>57</sup> and is the key mediator of canonical Wnt signaling, was detected in the cell membrane of undifferentiated and mature corneal epithelial cells. Nuclear localization of  $\beta$ -catenin was detected in a small subset of basal epithelial cells in the limbus, which indicates that Wnt/ $\beta$ -catenin signaling is active (Fig. 1D, inset). Kawakita et al.<sup>29</sup> reported  $\beta$ -catenin nuclear localization in limbal basal epithelial cells under air exposure and these cells were actively proliferating, as indicated by Ki67 expression. Their finding is consistent with our finding.

Interestingly, WIF1, a secreted protein that binds to Wnt molecules and inhibits Wnt signaling,<sup>58</sup> and FRZB were previously shown to be preferentially expressed at the limbus,<sup>27,30</sup> and our present study shows that WIF1 and FRZB are also upregulated in the human limbus (Fig. 1A). Furthermore, two other Wnt inhibitors, SFRP-5 and Dkk1, were upregulated in the limbus. In contrast, we did not detect any Wnt inhibitors that were specific to the central cornea. These findings are intriguing and suggest that Wnt signaling may be inhibited in LSCs under normal physiological conditions to induce a quiescent state despite the presence of several Wnt activators. In this scenario, only on removal of these Wnt inhibitors would Wnt signaling be activated to induce expression of down-



**FIGURE 4.** The undifferentiated state of LSCs was maintained when Wnt/ $\beta$ -catenin signaling was activated. (A–F) Expressions of ABCG2,  $\Delta Np63\alpha$ , and K12 were detected by immunostaining. There was high expression of ABCG2 and  $\Delta Np63\alpha$  in both control and LiCl-treated cultures (B, D). Expression of K12 in the control (E) was much higher compared with that in LiCl-treated cultures (F). (G) Relative mRNA expression levels quantified by qRT-PCR. Expression of ABCG2 was maintained and expression of  $\Delta Np63\alpha$  was significantly higher in LiCl-treated cultures. K12 expression was lower in the presence of LiCl. \* $P < 0.05$ . Scale bar, 20  $\mu$ m.

stream target genes. This concept is consistent with results from a study in keratinocyte stem cells in the human hair follicle bulge where two Wnt inhibitors, WIF1 and Dkk3, were upregulated, a result that also suggests repression of Wnt signaling in keratinocyte stem cells promotes quiescence.<sup>59</sup> This well-checked Wnt signaling is of great significance in preventing development of cancer from unregulated proliferation. Further studies are necessary to test this hypothesis.

LiCl-treated cultures yielded larger colonies that also contained increased numbers of limbal epithelial cells, a result that indicated a higher proliferation rate. This finding was confirmed by significantly increased expression of Ki67. Importantly, the expression levels of two putative LSC markers, ABCG2 and  $\Delta$ Np63 $\alpha$ , were preserved, and the expression of the maturation marker, K12, decreased. A significant increase in  $\Delta$ Np63 $\alpha$  mRNA expression was observed in LiCl-treated cultures compared with control (Fig. 4G). This specific isoform is proposed to be a more specific marker of LSCs.<sup>37–39</sup> There was also an increase in ABCG2 mRNA expression in the LiCl-treated LSCs, although the difference did not reach statistical significance. The intensity of the fluorescence in the immunohistochemistry study also slightly increased in the LiCl-treated group, but the overall intensity did not differ dramatically. Taken together, these results suggest that the activation of the Wnt/ $\beta$ -catenin signaling pathway promotes expansion of LSCs without inducing differentiation in vitro. However, there is a variation in the effectiveness of LiCl in the LSC cultures. Additional studies will be needed to further elucidate the exact role of individual limbal-specific Wnt proteins in the regulation of LSC proliferation and differentiation.

A higher CFE was observed in LSC cultures when Wnt/ $\beta$ -catenin signaling was activated. This increase may be attributed to a higher proliferative capacity of cells that do not normally proliferate in the current culturing system. The other possibility is that activation of Wnt/ $\beta$ -catenin signaling prevents premature apoptosis of LSCs in vitro. LSCs undergo apoptosis in culture when they are removed from their natural niche.<sup>60</sup> The niche factors provide crucial survival signals to stem cells in addition to regulating their proliferation and differentiation. Several papers have reported that Wnt/ $\beta$ -catenin signaling inhibits apoptosis in a variety of cell types.<sup>61–64</sup> It is possible that canonical Wnt signaling promotes cell proliferation and prevents cell death in cornea epithelial progenitor cells in vitro. Therefore, appropriate modulation of Wnt/ $\beta$ -catenin signaling has potential clinical application to increase the efficacy of ex vivo expansion of LSCs.

In conclusion, our findings demonstrate that the Wnt signaling pathway is present in ocular surface epithelial cells and that several Wnt activators and inhibitors are exclusive to the limbal region where cornea epithelial stem cells are located. Activation of Wnt/ $\beta$ -catenin signaling increases proliferation without inducing differentiation of cornea epithelial progenitor/stem cells in vitro. Taken together, our findings suggest a pivotal role of the Wnt signaling pathway in the regulation of LSC proliferation.

### Acknowledgments

The authors thank David Ha and Daniel Vu for technical assistance and Debora Farber for use of the real-time Stratagene PCR system.

### References

- Davanger M, Evensen A. Role of the pericorneal papillary structure in renewal of corneal epithelium. *Nature*. 1971;229:560–561.
- Schermer A, Galvin S, Sun TT. Differentiation-related expression of a major 64K corneal keratin in vivo and in culture suggests limbal location of corneal epithelial stem cells. *J Cell Biol*. 1986;103:49–62.
- Lehrer MS, Sun TT, Lavker RM. Strategies of epithelial repair: modulation of stem cell and transit amplifying cell proliferation. *J Cell Sci*. 1998;111:2867–2875.
- Lavker RM, Tseng SC, Sun TT. Corneal epithelial stem cells at the limbus: looking at some old problems from a new angle. *Exp Eye Res*. 2004;78:433–446.
- Cotsarelis G, Cheng SZ, Dong G, Sun TT, Lavker RM. Existence of slow-cycling limbal epithelial basal cells that can be preferentially stimulated to proliferate: implications on epithelial stem cells. *Cell*. 1989;57:201–209.
- Chen JJ, Tseng SC. Abnormal corneal epithelial wound healing in partial-thickness removal of limbal epithelium. *Invest Ophthalmol Vis Sci*. 1991;32:2219–2233.
- Lavker RM, Dong G, Cheng SZ, Kudoh K, Cotsarelis G, Sun TT. Relative proliferative rates of limbal and corneal epithelia. Implications of corneal epithelial migration, circadian rhythm, and suprabasally located DNA-synthesizing keratinocytes. *Invest Ophthalmol Vis Sci*. 1991;32:1864–1875.
- Pellegrini G, Traverso CE, Franzi AT, Zingirian M, Cancedda R, De Luca M. Long-term restoration of damaged corneal surfaces with autologous cultivated corneal epithelium. *Lancet*. 1997;349:990–993.
- Rama P, Matuska S, Paganoni G, Spinelli A, De Luca M, Pellegrini G. Limbal stem-cell therapy and long-term corneal regeneration. *N Engl J Med*. 2010;363:147–155.
- Tsai RJ, Li LM, Chen JK. Reconstruction of damaged corneas by transplantation of autologous limbal epithelial cells. *N Engl J Med*. 2000;343:86–93.
- Schwab IR, Reyes M, Isseroff RR. Successful transplantation of bioengineered tissue replacements in patients with ocular surface disease. *Cornea*. 2000;19:421–426.
- Fuchs E, Tumber T, Guasch G. Socializing with the neighbors: stem cells and their niche. *Cell*. 2004;116:769–778.
- Moore KA, Lemischka IR. Stem cells and their niches. *Science*. 2006;311:1880–1885.
- Li W, Hayashida Y, Chen YT, Tseng SC. Niche regulation of corneal epithelial stem cells at the limbus. *Cell Res*. 2007;17:26–36.
- Espana EM, Kawakita T, Romano A, et al. Stromal niche controls the plasticity of limbal and corneal epithelial differentiation in a rabbit model of recombined tissue. *Invest Ophthalmol Vis Sci*. 2003;44:5130–5135.
- Kolli S, Lako M, Figueiredo F, Mudhar H, Ahmad S. Loss of corneal epithelial stem cell properties in outgrowths from human limbal explants cultured on intact amniotic membrane. *Regen Med*. 2008;3:329–342.
- Blazewski EA, Schlotzer-Schrehardt U, Zenkel M, et al. Corneal limbal microenvironment can induce transdifferentiation of hair follicle stem cells into corneal epithelial-like cells. *Stem Cells*. 2009;27:642–652.
- Nusse R. Wnt signaling and stem cell control. *Cell Res*. 2008;18:523–527.
- Katoh M. Networking of WNT, FGF, Notch, BMP, and Hedgehog signaling pathways during carcinogenesis. *Stem Cell Rev*. 2007;3:30–38.
- Katoh M. WNT signaling pathway and stem cell signaling network. *Clin Cancer Res*. 2007;13:4042–4045.
- Das AV, Zhao X, James J, Kim M, Cowan KH, Ahmad I. Neural stem cells in the adult ciliary epithelium express GFAP and are regulated by Wnt signaling. *Biochem Biophys Res Commun*. 2006;339:708–716.
- Inoue T, Kagawa T, Fukushima M, et al. Activation of canonical Wnt pathway promotes proliferation of retinal stem cells derived from adult mouse ciliary margin. *Stem Cells*. 2006;24:95–104.
- Kubo F, Takeichi M, Nakagawa S. Wnt2b controls retinal cell differentiation at the ciliary marginal zone. *Development*. 2003;130:587–598.
- Lyu J, Joo CK. Wnt signaling enhances FGF2-triggered lens fiber cell differentiation. *Development*. 2004;131:1813–1824.
- Stump RJ, Ang S, Chen Y, et al. A role for Wnt/ $\beta$ -catenin signaling in lens epithelial differentiation. *Dev Biol*. 2003;259:48–61.

26. Figueira EC, Di Girolamo N, Coroneo MT, Wakefield D. The phenotype of limbal epithelial stem cells. *Invest Ophthalmol Vis Sci.* 2007;48:144–156.
27. Kulkarni BB, Tighe PJ, Mohammed I, et al. Comparative transcriptional profiling of the limbal epithelial crypt demonstrates its putative stem cell niche characteristics (Abstract). *BMC Genomics.* 2010;11:526.
28. Mukhopadhyay M, Gorivodsky M, Shtrom S, et al. Dkk2 plays an essential role in the corneal fate of the ocular surface epithelium. *Development.* 2006;133:2149–2154.
29. Kawakita T, Espana EM, He H, Li W, Liu CY, Tseng SC. Intrastromal invasion by limbal epithelial cells is mediated by epithelial-mesenchymal transition activated by air exposure. *Am J Pathol.* 2005;167:381–393.
30. Ding Z, Dong J, Liu J, Deng SX. Preferential gene expression in the limbus of the vervet monkey. *Mol Vis.* 2008;14:2031–2041.
31. Yoshida S, Shimmura S, Kawakita T, et al. Cytokeratin 15 can be used to identify the limbal phenotype in normal and diseased ocular surfaces. *Invest Ophthalmol Vis Sci.* 2006;47:4780–4786.
32. Spencer GJ, Utting JC, Etheridge SL, Arnett TR, Genever PG. Wnt signalling in osteoblasts regulates expression of the receptor activator of NF $\kappa$ B ligand and inhibits osteoclastogenesis in vitro. *J Cell Sci.* 2006;119:1283–1296.
33. Stambolic V, Ruel L, Woodgett JR. Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. *Curr Biol.* 1996;6:1664–1668.
34. de Paiva CS, Chen Z, Corrales RM, Pflugfelder SC, Li DQ. ABCG2 transporter identifies a population of clonogenic human limbal epithelial cells. *Stem Cells.* 2005;23:63–73.
35. Chen Z, de Paiva CS, Luo L, Kretzer FL, Pflugfelder SC, Li DQ. Characterization of putative stem cell phenotype in human limbal epithelia. *Stem Cells.* 2004;22:355–366.
36. Pellegrini G, Dellambra E, Golisano O, et al. p63 identifies keratinocyte stem cells. *Proc Natl Acad Sci USA.* 2001;98:3156–3161.
37. Kawasaki S, Tanioka H, Yamasaki K, Connon CJ, Kinoshita S. Expression and tissue distribution of p63 isoforms in human ocular surface epithelia. *Exp Eye Res.* 2006;82:293–299.
38. Di Iorio E, Barbaro V, Ruzza A, Ponzin D, Pellegrini G, De Luca M. Isoforms of DeltaNp63 and the migration of ocular limbal cells in human corneal regeneration. *Proc Natl Acad Sci USA.* 2005;102:9523–9528.
39. Barbaro V, Testa A, Di Iorio E, Mavilio F, Pellegrini G, De Luca M. C/EBPdelta regulates cell cycle and self-renewal of human limbal stem cells. *J Cell Biol.* 2007;177:1037–1049.
40. Lyu J, Joo CK. Expression of Wnt and MMP in epithelial cells during corneal wound healing. *Cornea.* 2006;25:S24–S28.
41. Lyu J, Joo CK. Wnt-7a up-regulates matrix metalloproteinase-12 expression and promotes cell proliferation in corneal epithelial cells during wound healing. *J Biol Chem.* 2005;280:21653–21660.
42. Sato N, Meijer L, Skaltsounis L, Greengard P, Brivanlou AH. Maintenance of pluripotency in human and mouse embryonic stem cells through activation of Wnt signaling by a pharmacological GSK-3-specific inhibitor. *Nat Med.* 2004;10:55–63.
43. Cai L, Ye Z, Zhou BY, Mali P, Zhou C, Cheng L. Promoting human embryonic stem cell renewal or differentiation by modulating Wnt signal and culture conditions. *Cell Res.* 2007;17:62–72.
44. Dravid G, Ye Z, Hammond H, et al. Defining the role of Wnt/beta-catenin signaling in the survival, proliferation, and self-renewal of human embryonic stem cells. *Stem Cells.* 2005;23:1489–1501.
45. Anton R, Kestler HA, Kuhl M. Beta-catenin signaling contributes to stemness and regulates early differentiation in murine embryonic stem cells. *FEBS Lett.* 2007;581:5247–5254.
46. Kalani MY, Cheshier SH, Cord BJ, et al. Wnt-mediated self-renewal of neural stem/progenitor cells. *Proc Natl Acad Sci USA.* 2008;105:16970–16975.
47. Lie DC, Colamarino SA, Song HJ, et al. Wnt signalling regulates adult hippocampal neurogenesis. *Nature.* 2005;437:1370–1375.
48. Huelsken J, Vogel R, Erdmann B, Cotsarelis G, Birchmeier W. beta-Catenin controls hair follicle morphogenesis and stem cell differentiation in the skin. *Cell.* 2001;105:533–545.
49. Zhu AJ, Watt FM. beta-catenin signalling modulates proliferative potential of human epidermal keratinocytes independently of intercellular adhesion. *Development.* 1999;126:2285–2298.
50. Zhou P, Byrne C, Jacobs J, Fuchs E. Lymphoid enhancer factor 1 directs hair follicle patterning and epithelial cell fate. *Genes Dev.* 1995;9:700–713.
51. Fevr T, Robine S, Louvard D, Huelsken J. Wnt/beta-catenin is essential for intestinal homeostasis and maintenance of intestinal stem cells. *Mol Cell Biol.* 2007;27:7551–7559.
52. Pinto D, Gregorieff A, Begthel H, Clevers H. Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. *Genes Dev.* 2003;17:1709–1713.
53. Fleming HE, Janzen V, Lo Celso C, et al. Wnt signaling in the niche enforces hematopoietic stem cell quiescence and is necessary to preserve self-renewal in vivo. *Cell Stem Cell.* 2008;2:274–283.
54. Luis TC, Weerkamp F, Naber BA, et al. Wnt3a deficiency irreversibly impairs hematopoietic stem cell self-renewal and leads to defects in progenitor cell differentiation. *Blood.* 2009;113:546–554.
55. Malhotra S, Baba Y, Garrett KP, Staal FJ, Gerstein R, Kincade PW. Contrasting responses of lymphoid progenitors to canonical and noncanonical Wnt signals. *J Immunol.* 2008;181:3955–3964.
56. Nemeth MJ, Topol L, Anderson SM, Yang Y, Bodine DM. Wnt5a inhibits canonical Wnt signaling in hematopoietic stem cells and enhances repopulation. *Proc Natl Acad Sci USA.* 2007;104:15436–15441.
57. Nelson WJ, Nusse R. Convergence of Wnt, beta-catenin, and cadherin pathways. *Science.* 2004;303:1483–1487.
58. Hsieh JC, Kodjabachian L, Rebbert ML, et al. A new secreted protein that binds to Wnt proteins and inhibits their activities. *Nature.* 1999;398:431–436.
59. Ohyama M, Terunuma A, Tock CL, et al. Characterization and isolation of stem cell-enriched human hair follicle bulge cells. *J Clin Invest.* 2006;116:249–260.
60. Sun CC, Su Pang JH, Cheng CY, et al. Interleukin-1 receptor antagonist (IL-1RA) prevents apoptosis in ex vivo expansion of human limbal epithelial cells cultivated on human amniotic membrane. *Stem Cells.* 2006;24:2130–2139.
61. Chen S, Guttridge DC, You Z, et al. Wnt-1 signaling inhibits apoptosis by activating beta-catenin/T cell factor-mediated transcription. *J Cell Biol.* 2001;152:87–96.
62. He B, Reguart N, You L, et al. Blockade of Wnt-1 signaling induces apoptosis in human colorectal cancer cells containing downstream mutations. *Oncogene.* 2005;24:3054–3058.
63. Reguart N, He B, Taron M, You L, Jablons DM, Rosell R. The role of Wnt signaling in cancer and stem cells. *Future Oncol.* 2005;1:787–797.
64. Yang F, Zeng Q, Yu G, Li S, Wang CY. Wnt/beta-catenin signaling inhibits death receptor-mediated apoptosis and promotes invasive growth of HNSCC. *Cell Signal.* 2006;18:679–687.